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by

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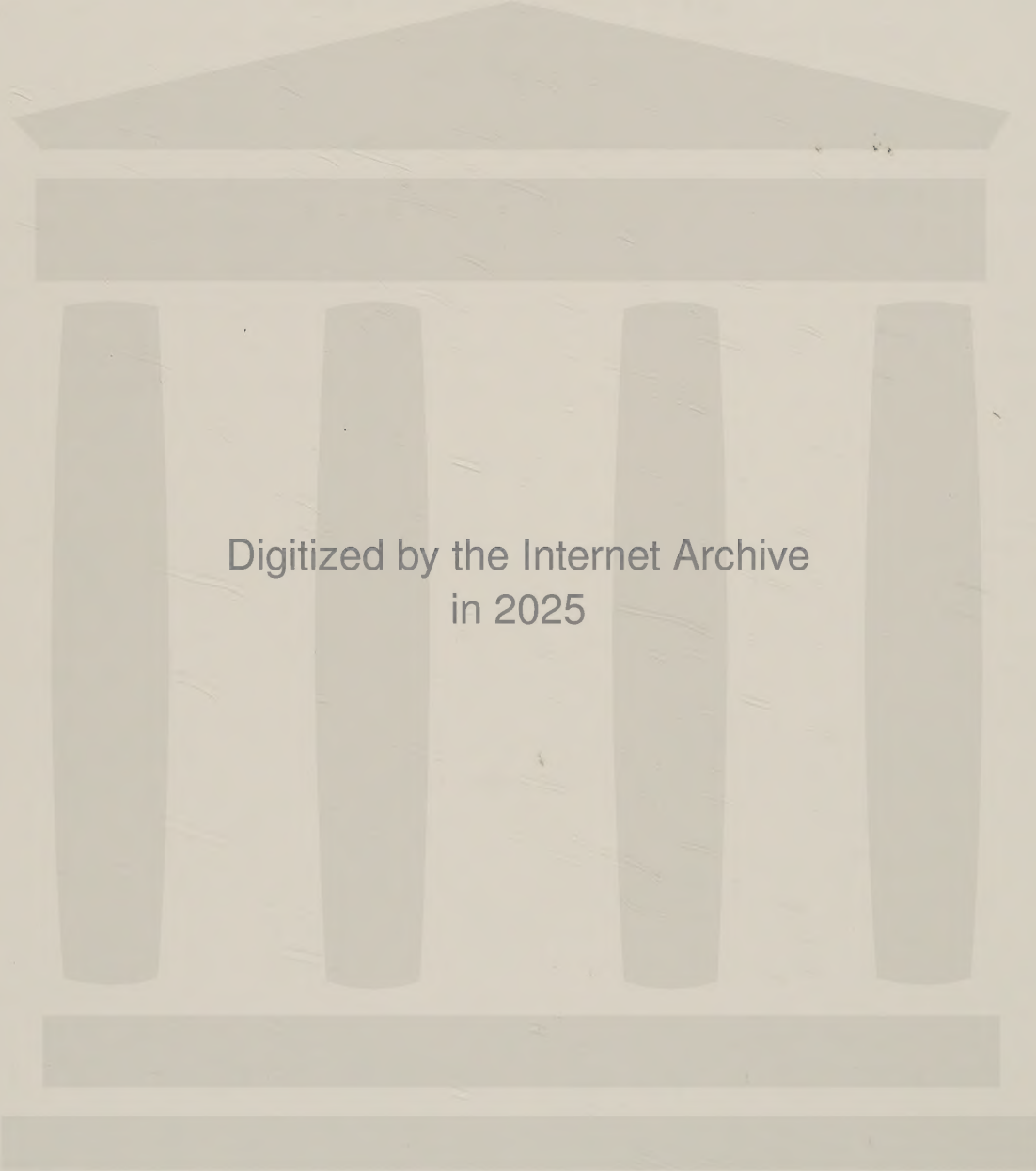
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**GENETIC RELATEDNESS WITHIN GROUPS
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INTRODUCTION

Accurate definitions of stock boundaries depend on unbiased estimates of population structure. A potentially confounding factor in studies of stock structure in sperm whales is that females and their young travel in groups. The statistical tests of heterogeneity used to assess population structure (such as analyses of variance) assume that samples are independent. Any data set containing related individuals violates this assumption. Thus, using samples of related individuals taken from these sperm whale groups would cause estimates of population structure to be positively biased, leading to false conclusions of population structure. Despite the fact that many species travel with kin, little work has been done to estimate or eliminate the degree of bias introduced when multiple samples are taken from social groups. Lyrholm et al. (1999) eliminated this bias by sampling only one individual from each group. Unfortunately, because sample size is greatly reduced, the statistical power to detect population structure is also greatly decreased. Here, we present a new method that removes related individuals from data sets, while retaining the maximum sample size possible. We use data from microsatellites to estimate relatedness. We then cull related individuals from social groups sampled in the eastern North Pacific and then use the remaining "unrelated" individuals to address stock boundaries in the area affected by the CA drift-net fishery.

Because related individuals have been removed, we are able to use both mitochondrial DNA (mtDNA) and microsatellites as separate genetic markers to examine stock structure. Because mtDNA is inherited as a unit from mother to offspring, it is commonly used successfully in delineating stock structure. In the case of sperm whales, however, diversity is inexplicably low (Richard et al. 1996, Lyrholm et al. 1996, Dillon 1996). Most animals world-wide belong to one of three haplotypes. Lack of variability can result in low statistical power to detect population structure. For example, many early studies that couldn't find population structure using allozymes are now finding structure using more variable loci like mtDNA. Thus, in addition to using microsatellites to estimate relatedness, we use the same data as a more variable genetic marker to investigate population structure.

METHODS

Sample Collection. Tissue samples from 348 sperm whales were obtained by SWFSC from strandings, incidental fishery takes, biopsy of live animals or from sloughed skin (Figure 1). Samples from the existing management units (coastal waters of California, Oregon and Washington, $n = 17$; Hawaii, $n = 4$; and AK, $n = 4$) are summarized in Table 1. Data from an additional 163 samples obtained from Japanese pelagic whaling operations in the North Pacific during 1978 (the eastern samples) and 1983-1984 (the western samples) were provided by Thomas Lyrholm (contract report to SWFSC 1997).

Mitochondrial DNA. For the mtDNA analyses, 401 base pairs of the control region of the mitochondrial genome were amplified using a primer that annealed to a portion of the tRNA threonine and the tRNA proline gene and another primer (Pmac, designed for this study) that annealed to a conserved section within the control region. The samples were run on an ABI autosequencer and aligned by eye. Hypotheses of population structure were tested using an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN (Excoffier et al 1992). AMOVA calculates fixation indices based on frequency (F_{st}) and both frequency and genetic distance (Φ_{st}).

When allele diversity is low, i.e. many individuals share the same haplotypes, frequency based statistics (F_{st}) have higher statistical power than distance based statistics (Φ_{st}). Because sperm whales are known to have low haplotypic diversity in mtDNA (Richard et al. 1996, Lyrholm et al. 1996, Dillon 1996), we expect F_{st} to be the more powerful statistic.

Microsatellites. For microsatellite analysis, six to nine microsatellite (dinucleotide) loci were amplified. PCR products were run on an ABI autosequencer with an internal size standard for allelic determination and analyzed with Genescan Analysis Software. Significant allele frequency differences among groups were calculated using an F-statistic implemented in the computer program Arlequin.

Relatedness. We used microsatellite data to estimate (Kinship version 1.2; Goodnight et al.) pairwise coefficients of relatedness among all individuals sampled within social groups (defined as those whales sampled on the same day, in close association and with more or less coordinated movements). These pairwise estimates were then entered into a custom written True Basic program ("Kin-Be-Gone"; Taylor unpublished). The program selectively removes the minimum number of individuals such that all remaining individuals have pairwise coefficients of relatedness less than 0.25, which is the mean value expected for second order relatives. The output is a list of "unrelated" individuals for subsequent analyses of population structure.

RESULTS

MtDNA. Twenty nucleotide sites out of 401 were variable among the 535 sperm whale sequences, identifying 23 mtDNA haplotypes (Table 2). Eighty-four percent of the individuals typed had one of three haplotypes (Table 2). Nine of the 23 haplotypes (39%) are present in the coastal waters of California, Oregon and Washington (Table 1).

Microsatellites. The number of alleles/locus ranged from 11-22. The putative populations shared similar common microsatellite alleles and genotypes but also showed a few unique alleles/haplotypes (data available upon request). Many of the loci showed statistically significant deviations from Hardy Weinberg equilibrium in at least one putative population (this may indicate inbreeding, the presence of null alleles, or the lumping of separate populations in the analysis).

Relatedness. To demonstrate the potential bias introduced by using related individuals in population structure analyses, we calculated measures of genetic differentiation for groups of stranded individual that contained known relatives (such as mother/fetus pairs). We then removed the related individuals as described above and compared the groups that now consisted only of unrelated individuals. Comparisons between AMOVA results (Table 3) calculated with "all individuals" and "kin-be-gone" data sets illustrate the positive bias that may be introduced into population analyses when multiple samples are obtained from groups of related individuals ($p < 0.001$ for "all individuals" and $p = 0.641$ for "kin-be-gone" individuals).

Population subdivision. Analyses of molecular variance (AMOVA) showed statistically significant genetic subdivision (Table 4) between samples collected from the coastal waters of CA/OR/WA and samples collected in the following three areas of the eastern North Pacific (see Figure 1):

- (1) "Eastern" (Japanese pelagic whaling samples obtained in an area between 24° - 31° North and 120° - 129° East which is an area roughly 300 - 500nm and distributed between San Diego and Cabo San Lucas),
- (2) "Central" (includes "eastern" and samples collected out to Hawaii; includes Japanese pelagic whaling and samples obtained from the dedicated SWFSC cruise, "SWAPS" in 1997), and
- (3) Gulf of California (samples from SWFSC cruises ("CADDIS" 1995, "VAQUITA" 1997) and two stranding events in Bahia La Paz in 1993 and 1997).

DISCUSSION

Our results show statistically significant east-west subdivision between coastal California, Oregon and Washington and areas offshore to Hawaii. For the first time, we have also found statistically significant north-south subdivision between coastal California, Oregon and Washington and the Gulf of California. As expected, significant structure was found primarily using microsatellite data. Only in the case with the highest sample size (from the central eastern North Pacific) was a significant difference found using mtDNA. Again, statistical power is expected to be low because diversity in this marker is not only low but most individuals belong to one of three haplotypes (Table 1). Although, these results support the hypothesis that sperm whales from coastal waters off California, Oregon and Washington represent a separate stock, caution is warranted. While we are making progress in coping with the bias introduced by related individuals, further progress in determining stock boundaries for the area affected by the fishery is hindered by a paucity of samples. Although we have a large scientific collection of sperm whale tissue samples from the Pacific, Indian and Atlantic Ocean on hand (currently $n = 500+$) we have less than 20 usable samples from the coastal waters off California, Oregon and Washington. We have even fewer samples to the north in Canadian, Alaskan and adjacent offshore waters. Because the only recaptures of discovery tags placed in sperm whales off San Francisco were made in these waters it is clear that future work should be directed towards increasing samples from these areas.

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Table 1. SWFSC Pnac samples from the existing management units and adjacent areas. 22 Nov 99

REGION	LABID	Date	SOURCE	SITE	Disposition of Sample	Group	size	SEX	MDNA	M-sat
CAORWA - 1	75	24-May-93	STRAND-CA-LACM72545	Laguna Niguel, CA	stranding	single	4.40m	m	J	1: no tissue left but now have sample 10403
CAORWA - 2	2181	20-Dec-93	CAGN	Southern CA	drift net - incidental take	single	12.19m	m	H	2
CAORWA - 3	2364	29-Apr-94	STRAND-CA-LACM8993	Cambria, CA	stranding	single	5.47m	f	C	3
CAORWA - 4	2365	01-May-94	STRAND-CA-LACM	Pt Sal, CA	stranding	single	4.15	m	B	failed previous - trying again w/ little luck
CAORWA - omit	5607	20-Dec-72	STRAND-CA-LACM54609	Camp Pendleton, CA	stranding	single	18.23m	m	fail	failed previous - trying again w/ little luck
CAORWA - omit	5752	21-Jul-96	BIOPSY-PT 873-ORCAWA	off Newport, Oregon	biopsy - no tissue left	group 1 - ok	u	u	fail	no tissue left
CAORWA - 5	5753	21-Jul-96	BIOPSY-PT 873-ORCAWA	off Newport, Oregon	biopsy	group 1 - ok	u	m	A	4
CAORWA - 6	5754	21-Jul-96	BIOPSY-PT 873-ORCAWA	off Newport, Oregon	biopsy	group 1 - ok	u	m	E	5
CAORWA - omit	6180	14-Sep-96	BIOPSY-PT 873-ORCAWA	off Lopez Point (Big Sur), CA	biopsy - no tissue left	single	u	u	fail	no tissue left
CAORWA - 7	6181	15-Sep-96	BIOPSY-PT 873-ORCAWA	off Point Sur, CA	biopsy	single	u	f	B	6
CAORWA - 8	6223	10-Oct-96	BIOPSY-PT 873-ORCAWA	off Cape Mendocino, CA	biopsy	single	u	m	I	7
CAORWA - 9	6275	08-Sep-96	BIOPSY-PT 873-ORCAWA	Oregon/Washington border	stranding	single	4.25m	m	B	8
CAORWA - 10	7644	17-Jul-97	STRAND-CA-TMMC	Ano Nuevo, CA	stranding	single	11.9m	u	M	9
CAORWA - 11	8518	06-Oct-97	STRAND-OR-OIMB	Coos Bay, Oregon	stranding	single	11.9m	u	K	10
CAORWA - omit	8624	19-Oct-97	BIOPSY-PT 873-SPERM	Santa Lucia Escarpment	sloughed skin	group 27 - omit	large	m	A (omit)	omit (related)
CAORWA - 12	8625	20-Oct-97	BIOPSY-PT 873-SPERM	Santa Lucia Escarpment - Grp A	skin collected from tagging attempt	group 27 - ok	large	f	B	11
CAORWA - omit	8626	20-Oct-97	BIOPSY-PT 873-SPERM	Santa Lucia Escarpment - Grp A	sloughed skin	group 27 - omit	medium	u	B (omit)	omit (related)
CAORWA - 14	8627	21-Oct-97	BIOPSY-PT 873-SPERM	Santa Lucia Escarpment	flesh floating from Orca attack	group 27 - ok	large	f	A	12
CAORWA - 15	8629	26-Oct-97	BIOPSY-PT 873-SPERM	Santa Lucia Escarpment	sloughed skin	group 27 - ok	large	f	C	13
CAORWA - omit	10403 = 75	24-May-93	STRAND-CA-LACM72545	Laguna Niguel, CA	stranding - jerky	single	4.40m	m	omit	10403 = 75
CAORWA - wait	10404	02-May-94	STRAND-CA-LACM89932	Pismo Beach	stranding - spermicell	single	11.15m	f	fail	John - how about some skin?
CAORWA - 16	12147	17-Nov-98	CAGN	off Lopez Point, Big Sur, CA	drift net - incidental take	single	u	m	A	processing
CAORWA - 17	12306	30-Jan-99	STRAND-CA-TMMC	Cambria, CA	stranding	single	14.7m	m	B	processing
AK - 1	4848	1995	STRAND-AK-UAF-WYNNE	Copper River Delta/Kodiak, AK	stranding	single	16m	m	B	1
AK - 2	10042	1994	STRAND-AK-NMFS	Attu Island, AK	stranding - bone collected from teeth sockets	single	u	f?	B	will do
AK - 3	10399	10-Jun-98	AK-FISHERY	south of Seward, Gulf of Alaska	biopsy	group 3	adult	u	C	2
AK - 4	10400	10-Jun-98	AK-FISHERY	south of Seward, Gulf of Alaska	biopsy	group 3	adult	u	A	3
CANADA - 1	11218	01-May-98	CANADA-LENNARD	Estevan Point, West Vancouver Is, Canada	stranding	single	17.4m	m	B	1
CANADA - 2	11219	1997	CANADA-LENNARD	west end of Johnston Strait, Canada	stranding	single	ad	u	A	will do
CANADA - 3	13668	09-Jul-99	CANADA-LENNARD	Hawquilt Harbor, West Vancouver Is, Canada	stranding	single	5.6m	f	processing	will do
W BAJA - 1	4628	12-Sep-95	BIOPSY-PT 873-CADDIS	west of Baja	biopsy	single	u	m	C	1
W BAJA - 2	11535	04-Aug-98	SPAM98-MEXICO	west of Baja California, Mexico	sloughed skin	group 4	subadult	u	processing	2
W BAJA - 3	11536	04-Aug-98	SPAM98-MEXICO	west of Baja California, Mexico	sloughed skin	group 4	subadult	u	G	3
W BAJA - 4	11537	04-Aug-98	SPAM98-MEXICO	west of Baja California, Mexico	biopsy	group 4	subadult	u	S	4
W BAJA - 5	11538	04-Aug-98	SPAM98-MEXICO	west of Baja California, Mexico	biopsy	group 4	large	u	C	5
HI - 1	9712	02-Dec-97	STRAND-HI-NMFS	Oahu, HI	stranding	single	u	u	B	will by
HI - omit	7545	02-Apr-97	BIOPSY-PT 873-SWAPS	south of Oahu, HI	biopsy	group 5 - omit	u	f	A	omit (related)
HI - 2	7546	02-Apr-97	BIOPSY-PT 873-SWAPS	south of Oahu, HI	biopsy	group 5 - ok	u	f	A	1
HI - 3	7547	09-Apr-97	BIOPSY-PT 873-SWAPS	south of Oahu, HI	biopsy	group 6 - ok	u	f	B	2
HI - 4	7548	09-Apr-97	BIOPSY-SWAPS-SLGH-SK	south of Oahu, HI	sloughed skin	group 6 - ok	u	f	B	3

Table 3. Comparison of AMOVA results between all individuals and culled-for-unrelated individuals. Analysis of molecular variance (AMOVA) for microsatellite data among the three Tasmanian strandings (n = 64, 37 and 11). Pairwise comparisons (F_{st}) between populations and significance values (probability of random values being greater than the observed value). The number of iterations = 1000. The “culled-for-unrelated” removes all individuals from the analysis that have a coefficient of relatedness equal to or greater than 0.25 (the mean value expected for second order relations).

3a. All Stranded Animals

Source of Variation	d.f.	Percentage of Variation	F_{st}	p
Among Stranded Groups	2	1.75	0.017	0.000
Within Stranded Groups	221	98.25	-----	-----

3b. “Kin-be-gone”

Source of Variation	d.f.	Percentage of Variation	F_{st}	p
Among Stranded Groups	2	-0.231	-0.002	0.641
Within Stranded Groups	89	100.23	-----	-----

Table 4. Eastern North Pacific, four putative populations. Analysis of molecular variance (AMOVA) for mtDNA data and microsatellite data after removing individuals with coefficients of relatedness > 0.25 via the program "kin-be-gone" (B. Taylor, unpublished). Pairwise comparisons (Φ_{st} and F_{st}) among populations and significance values (probability of random values being greater than the observed value). Statistically significant genetic subdivision between populations is indicated in bold face. The number of iterations = 1000. Data from SWFSC and T. Lytholm.

	"central" (MtDNA n = 40, msat n = 48)	"eastern" (MtDNA n = 9, msat n = 16)	Gulf of CA (mtDNA n = 28, msat n = 28)
CA/OR/WA (MtDNA n = 16) (msat n = 14)	$\Phi_{st} = 0.055,$ $F_{st} = 0.096,$ msat $F_{st} = 0.026,$ $p = 0.046$ $p = 0.006$ $p = 0.009$	$\Phi_{st} = -0.031,$ $F_{st} = 0.005,$ msat $F_{st} = 0.078,$ $p = 0.754$ $p = 0.329$ $p < 0.001$	$\Phi_{st} = -0.017,$ $F_{st} = -0.004,$ msat $F_{st} = 0.064,$ $p = 0.631$ $p = 0.439$ $p = 0.005$

